Faculty of Medicine and Health Sciences

Optimisation of PC12 Cell-based *In Vitro* Stroke Model for Screening of Potential Neuroprotective Agents

Chua Pin Fen

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DECLARATION

I hereby declare that this thesis is my original work. All sources of information which were used as references to compose this thesis have been acknowledged within the text. The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

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Chua Pin Fen

Molecular Pharmacology Lab

Faculty of Medicine and Health Sciences

Universiti Malaysia Sarawak

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ABSTRACT

Stroke is one of the commonest causes of death and disability worldwide. To date, no neuroprotective agent is approved clinically for treatment of acute ischaemic stroke. PC12 cell-based *in vitro* stroke model has been used widely to screen for neuroprotective agents. The most common approach is to differentiate PC12 cells into a neuronal phenotype and subject the differentiated cells to oxygen glucose deprivation (OGD) to simulate an ischaemic stroke. However, there are increasing reports showing certain PC12 cell variants either poorly or cannot differentiate into neuron-like cells. Additionally, optimisation studies for cell culture media and adhesive substrate for commonly-used PC12 variants are limited. This study aimed to optimise cell culture conditions of three commonly-used PC12 cell variants and test their response to nerve growth factor (NGF)-induced differentiation. PC12 cells (Riken cell bank), PC12 Adh cells and PC12 Neuroscreen-1 (NS-1) cells were studied. Optimisation of cell culture media and adhesive substrates were done by comparing cell morphology in different media and substrates. Adaptation of the three PC12 cell variants to a serum-free supplemented media was necessary because drug intervention studies need to be done in the absence of serum to avoid confounding effects. Comparison of the NGF-induced differentiation for the three PC12 cell variants was done by counting the percentage of neurite-bearing cells. The PC12 variant with the highest response to NGF underwent studies to optimise its duration of OGD. Differentiated-cells were subjected to different durations of OGD followed by measurement of cell viability and enzyme activity in the execution phase of apoptosis (caspase 3 and 7) to determine the optimal duration of OGD for *in vitro* stroke model. The *in vitro* model was validated by treating the OGD-treated cells with a known neuroprotectant, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) for

24 hours. The optimal culture media for PC12 cells (Riken) and NS-1 cells was found to be DMEM high glucose. Collagen IV was the best substrate for PC12 cells (Riken) and NS-1 cells. PC12 Adh cells showed no preference for media or substrates. Both NS-1 cells and PC12 cells (Riken) were successfully adapted to serum-free supplemented media. However, PC12 Adh cells failed to grow in a serum-free supplemented media. NS-1 cells gave the highest NGF-induced differentiation (72.7 ± 9.1%) followed by PC12 cells (Riken, 36.0 ± 5.6%) and PC12 Adh cells (6.9 ± 0.8%). NS-1 cells achieved optimal differentiation after three days of 150 ng/mL NGF treatment. The optimal duration of OGD to induce cell injury was three hours. Treatment with 8-OH-DPAT on NGF-differentiated NS-1 cells after three hours of OGD showed a significant reduction in apoptosis ($p < 0.01$, One-way ANOVA followed by post hoc Tukey's test). In conclusion, we have shown that each PC12 variant has differing requirements for media and adhesive substrates. NS-1 cells were the optimal PC12 cell variant for an *in vitro* stroke model due to its high level of NGF-induced differentiation. DMEM high glucose was the optimal media and collagen IV was the best substrate for NS-1 cells. We also provide the first report that 8-OH-DPAT is neuroprotective in NS-1 cells. We conclude that we have optimised and set up an *in vitro* stroke model to screen for potential neuroprotective agents.

Keywords: Stroke, PC12 cell, adhesive substrate, NGF-differentiation, oxygen deprivation assay, apoptosis assay, neuroprotective agents.

Pengoptimuman Model Strok In Vitro berasaskan Sel PC12 untuk Penyaringan Agen Neuroprotektif yang Berpotensi

ABSTRAK

Penyakit Strok adalah salah satu penyebab utama kematian dan kecacatan di seluruh tetapi masih tiada agen neuroprotektif diluluskan untuk rawatan strok iskemia. Model strok in vitro berasaskan sel PC12 telah digunakan secara meluas untuk penyaringan agen-agen neuroprotektif. Pendekatan yang paling lazim digunakan adalah membezakan sel PC12 kepada fenotip neuron dan mendedahkan sel tersebut kepada keadaaan kekurangan glukosa dan oksigen (OGD) untuk mensimulasikan strok iskemia. Walau bagaimanapun, terdapat peningkatan laporan yang menunjukkan varian sel PC12 tertentu yang tidak dapat atau kurang berupaya untuk berbeza kepada sel seperti neuron. Kajian pengoptimuman untuk media kultur sel dan substrat perekat untuk varian PC12 yang lazim digunakan adalah terhad. Kajian ini bertujuan untuk mengoptimumkan keadaan kultur sel untuk tiga varian sel PC12 dan menguji tindak balas mereka terhadap pembezaan dengan faktor pertumbuhan saraf (NGF). PC12 (bank sel Riken), PC12 Adh dan PC12 Neuroscreen-1 (NS-1) telah dikaji. Pengoptimuman media kultur sel dan substrat pelekat dilakukan dengan membandingkan morfologi tiga varian sel dalam media dan substrat pelekat yang berbeza. Penyesuaian varian sel tiga PC12 ke media tambahan tanpa serum adalah perlu kerana kajian ubat baru perlu dilakukan tanpa serum untuk mengelakkan kesan yang mengelirukan. Perbandingan tindak balas kepada pembezaan yang disebabkan oleh NGF untuk tiga varian sel telah dilakukan dengan mengira peratusan sel yang mengandungi neurit. Varian sel yang menunjukkan tindak balas tertinggi kepada NGF digunakan untuk mengoptimumkan tempoh OGD. Sel tersebut tertakluk kepada tempoh OGD yang berlainan dan diikuti dengan pengukuran daya aktif sel dan aktiviti enzim dalam fasa pelaksanaan apoptosis (caspase 3 dan 7) untuk menentukan tempoh optimum OGD. Model

strok in vitro disahkan dengan merawat sel yang telah dicedera oleh OGD dengan menggunakan agen neuroprotektif yang dikenali sebagai 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) selama 24 jam. Hasil kajian menunjukkan media kultur optimum untuk PC12 (Riken) dan NS-1 adalah DMEM tinggi gula. Kolagen IV adalah substrat terbaik untuk PC12 (Riken) dan NS-1. PC12 Adh tidak menunjukkan keutamaan dalam mana-mana media atau substrat. NS-1 dan PC12 (Riken) berjaya disesuaikan dengan media tambahan tanpa serum dan dapat hidup di media tersebut. Walau bagaimanapun, PC12 Adh gagal berkembang dalam media tambahan tanpa serum. NS-1 menghasilkan pembezaan yang disebabkan oleh NGF (72.7 ± 9.1%) yang tertinggi diikuti oleh PC12 (Riken, 36.0 ± 5.6%) dan PC12 Adh (6.9 ± 0.8%). Sel NS-1 mencapai pembezaan optimum selepas tiga hari rawatan 150 ng/mL NGF. Tempoh optimum OGD untuk merangsang kecederaan sel adalah tiga jam. Rawatan dengan 8-OH-DPAT pada sel NS-1 yang telah dibezakan selepas tiga jam OGD menunjukkan pengurangan apoptosis yang signifikan ($p < 0.01$) (satu arah ANOVA diikuti oleh ujian Tuki post hoc). Kesimpulannya, kami telah menunjukkan bahawa setiap varian PC12 mempunyai keperluan berbeza untuk media dan substrat pelekat. Kami melaporkan bahawa 8-OH-DPAT adalah neuroprotektif dalam NS-1 dengan kali pertama. NS-1 adalah varian sel PC12 yang optimum untuk model strok in vitro kerana mempunyai tahap perbezaan oleh faktor NGF yang paling tinggi berbanding dengan dua varian PC12 yang lain. DMEM tinggi gula adalah media optimum dan kolagen IV adalah substrat terbaik untuk NS-1. NS-1 yang didedahkan kepada tiga jam OGD menyerupai strok iskemia. Kesimpulannya, kami telah mengoptimumkan dan menubuhkan model strok in vitro untuk menyaring agen-agen neuroprotektif yang berpotensi.

***Kata kunci**: Strok, sel PC12, substrak pelekat, pembezaan dengan faktor pertumbuhan saraf, kekurangan glukosa dan oksigen, apoptosis, agen-agen neuroprotektif*

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
BD	Becton Dickinson
BSA	bovine serum albumin
CO_2	carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinases
FBS	fetal bovine serum
F-12K	Ham's F-12K (Kaighn's) Medium
HBSS	Hank’s balanced salt solution
HCL	hydrochloric acid
HS	horse serum
5-HT1A	5-hydroxytryptamine 1A receptors
mg	miligram

mL	mililitre
mM	milimolar
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
ng	nanogram
NGF	nerve growth factor
NIH	National Institutes of Health
nM	nanomolar
NS-1	Neuroscreen-1
OGD	oxygen glucose deprivation
OGD/R	oxygen glucose deprivation/reperfusion
8-OH-DPAT	8-hydroxy-2(di-n-propylamino) tetralin
PBS	phosphate buffered saline
PC	pheochromocytoma
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute
RTKs	receptor tyrosine kinase
SD	standard deviation

Trk	tyrosine kinases
μL	microlitre
μg	microgram
μM	micromolar
%	percentage
°C	degree Celcius

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Stroke

Stroke is the second commonest cause of death worldwide (Katan & Luft, 2018). Its global burden is high, inclusive of increasing mortality, incidence, disability-adjusted life year and economic impact, particularly in low- and middle-income countries (Mukherjee & Patil, 2011). It can cause cognitive and functional disabilities, contributing significantly to long term health care costs (Azad, Veeravagu, & Steinberg, 2016). In Malaysia, stroke is the third leading causes of death (Department of Statistic Malaysia, 2018). The incidence of local ischaemic stroke is on the trend of rising annually by 29.5% (Lee, Shafie, Sidek & Aziz, 2017). The prevalence of stroke was reported as 0.7%, with 1.7% among those aged 55-59 years, 2% in 60-64 years, 3% in 65-69 years, 3.5% in 70-74 years and 7.8% in 75 years and beyond in the fourth National Health and Morbidity Survey (NHMS) in 2011 (Ministry of Health Malaysia, 2011). Prognosis of the disease depends on many factors including age, stroke severity, infarct location, stroke mechanism, clinical findings and related complications. In addition, medical interventions for example thrombolysis, thrombectomy, stroke unit care, and rehabilitation influences the outcome of the disease.

1.1.1 Pathogenesis of Stroke

Stroke are caused by ischaemia (loss of blood supply) or haemorrhage (bleeding). Around 80% of stroke are caused by ischaemia. The most common ischaemic strokes include